SUBSTITUTE SPECIFICATION

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HIGHLY CONCENTRATED STABLIZED IgM SOLUTION

Cross-Reference to Related Applications

This application is the National Stage of International Application No. PCT/JP2004/014935, filed on October 8, 2004, which claims the benefit of Japanese Patent Application Serial No. 2003-351388, filed on October 9, 2003. The contents of both of the foregoing applications are hereby incorporated by reference in their entireties.

Technical Field

The present invention relates to highly concentrated and stabilized IgM solutions, and production thereof.

Background Art

Many higher animals have five different classes of immunoglobulins, IgG, IgA, IgM, IgD, and IgE. Each immunoglobulin class differs in properties such as size, charge, amino acid composition, and sugar content. Of these classes, IgM accounts for approximately 10% of all plasma immunoglobulins. IgM is the major component of early antibodies produced against cell-membrane antigens, infectious microorganisms, or soluble antigens, which have a complex antigenicity.

Human IgMs usually have a pentameric structure. Each of the five subunits constituting this pentameric structure has a four-stranded structure similar to that of IgG. The amino acid sequence of the μ chain, which is the heavy chain of IgM, is different from that of the γ chain, which is the heavy chain of IgG. The following differences can also be seen:

- The μ chain has an extra constant domain than the γ chain.
- The μ chain has four more oligosaccharide chains than the γ chain.

IgM has a polypeptide chain called the J chain, which is not found in IgG. The J chain is considered to assist the association of μ chains prior to secretion of IgM from antibody producing cells.

With advances in monoclonal antibody technology and recombinant DNA technology, large-scale production of pure immunoglobulins has become possible in recent years. Furthermore, gene recombination techniques have enabled production of chimeric antibodies and humanized antibodies. Chimeric antibodies are antibodies having a structure in which the variable regions have been replaced with variable regions derived from a different species. For example, "chimeric antibodies" comprising variable regions of non-human antibodies and the constant regions of human antibodies (Non-Patent Document 1/ Proc. Natl. Acad. Sci. U.S.A.,

(1984) 81:6851) are known. Also known are humanized antibodies in which the complementarity determining regions (CDR) of other animal species are transferred into human immunoglobulins (Non-Patent Document 2/ Nature (1986) 321:521).

Actual examples of antitumor antibodies are the anti-CD20 human chimeric antibody Rituxan (IDEC), and the anti-HER2/neu humanized antibody Herceptin (Genentech), which have completed clinical trials and have already been approved. These antibodies are now commercially available. Antibody-dependent cellular cytotoxicity (hereinafter referred to as ADCC) activity and complement-dependent cytotoxicity (hereinafter referred to as CDC) activity are known as effector functions of IgG and IgM. Since IgM has a higher CDC activity compared to IgG, it has an extremely high chance of becoming an anti-tumor antibody having CDC activity as its main effect. However, as described above, unlike IgG, IgM forms a multimer. Therefore, industrial scale production of recombinant IgM had been considered difficult.

IgM is also very unstable compared to IgG and has a low solubility. Therefore, the production of a highly concentrated and stable IgM solution is difficult. For example, Cytotherapy, 2001, 3(3), 233-242 (Non-Patent Document 5) reports that, even when IgM had been stored at -20°C, precipitation and decrease of activity occurred upon thawing. Furthermore, according to the report, IgM easily aggregates and precipitates during storage. It was especially difficult to ensure an IgM stability sufficient enough to withstand pharmaceutical use only through optimization of pH and buffer type.

Therefore, various attempts are being made to stabilize antibodies by methods other than optimization of pH and buffer type. For example, WO 2002/096457 (Patent Document 1) discloses formulations for stabilizing highly concentrated antibodies that comprise acidic ingredients. This method uses $MgCl_2$ and $CaCl_2$ as additives to stabilize the antibodies, but the stabilization is carried out to prepare IgG formulations, and IgM formulations are not mentioned. As described above, unlike IgG, IgM exists as a multimer, and unlike intrinsically stable IgG, IgM readily aggregates. Therefore, IgM has the distinctive problem of being very difficult to be highly concentrated.

Clin. Chem. Lab. Med. 2000; 38(8): 759-764 (Non-Patent Document 3) and Journal of Immunological Methods, 111 (1988) 17-23 (Non-Patent Document 5) reported that IgM precipitates at a low salt concentration, and redissolves at a high salt concentration in a phosphate buffer and Tris-hydrochloride buffer that are weakly alkaline. Clin. Chem. Lab. Med. 2000; 38(8): 759-764 (Non-Patent Document 3) reports that, near pH5, IgM readily precipitates and is difficult to handle, suggesting a pessimistic outlook for IgM solutions in weakly acidic buffers. This report thus gives no indication of the possibility of providing a highly concentrated IgM solution as a pharmaceutical or a bulk drug substance. This document also

reports that when human sera comprising a high concentration of IgM are diluted with water, insoluble aggregates are generated as euglobulin precipitates, increasing the turbidity of the solution; but when the salt concentration is then raised by adding NaCl, Arginine, or such, the euglobulin precipitates redissolve. However, this report relates to the reconstitution of euglobulin precipitates, and does not provide any disclosures relating to suppression of increase in water-soluble aggregates of IgM. Furthermore, since patient-derived unpurified sera comprising various serum proteins are used in this document, the resulting insoluble aggregates may comprise proteins other than IgM. The effects on IgM solution in the absence of the other proteins are not described.

In Journal of Immunological Methods, 111 (1988) 17-23 (Non-Patent Document 5), a buffer comprising 0.1 M Tris-HCl and 1 M NaCl (pH8) is used to redissolve euglobulin precipitates. However, the resulting recovery rate of IgM varies from 40% to >90% depending on antibodies or batch, indicating a low reproducibility. Additionally, although the Methods section describes that 5 to 10 mg/mL of purified antibodies were stored at 4°C and -20°C, the Results section only describes that the antibodies could be stored for a few months at -20°C without loss of function, and does not mention anything regarding storage at 4°C or higher, at which temperature it is usually difficult to ensure stability. Accordingly, this report suggests the difficulty of reproducing precipitate reconstitution and the difficulty of ensuring stability during storage, when trying to provide a highly concentrated solution of IgM as a pharmaceutical product or a bulk drug substance.

BIOTECHNOLOGY 1993, 11, 512-515 (Non-Patent Document 4) and Journal of Immunological Methods, 111 (1988) 17-23 (Non-Patent Document 5) also describe the reconstitution of insoluble aggregates of antibodies as euglobulin precipitates, but the solubility is 10 mg/mL or less, indicating low solubility of IgM. There is no description at all regarding the stabilization of water-soluble aggregates.

Pharmaceutical Research 1994, 11(5), 624-632 (Non-Patent Document 6) discloses stabilization of IgM by PVP addition, but does not disclose the stabilization of highly concentrated antibodies. Journal of Immunological Methods 1995, 181(1), 37-43 (Non-Patent Document 7) discloses lyophilized formulations produced by addition of trehalose, but in this report, the antibody stability is insufficient and there is no description relating to stabilization of highly concentrated antibodies.

Patent Document 1: WO 2002/096457
Non-Patent Document 1: Proc. Natl. Acad. Sci. U.S.A, (1984) 81:6851
Non-Patent Document 2: Nature (1986) 321:521
Non-Patent Document 3: Clin. Chem. Lab. Med. 2000; 38 (8):759-764

Non-Patent Document 4: BIOTECHNOLOGY 1993, 11, 512-515
Non-Patent Document 5: Journal of Immunological Methods, 111 (1988), 17-23
Non-Patent Document 6: Pharmaceutical Research 1994, 11(5), 624-632
Non-Patent Document 7: Journal of Immunological Methods 1995, 181, 37-43
Non-Patent Document 8: Cytotherapy, 2001, 3(3), 233-242

Disclosure of the Invention

Problems to be Solved by the Invention

The present invention was achieved in view of the above circumstances. An objective of the present invention is to stabilize highly concentrated IgM in solutions. More specifically, the present invention aims to provide methods for stabilizing highly concentrated IgM, solutions in which highly concentrated IgM is stabilized, and methods for preparing the solutions.

In a preferred embodiment of solutions in which highly concentrated IgM is stabilized, the present invention provides an aqueous solution in which increase of water-soluble aggregates is suppressed. Another preferred embodiment provides a highly concentrated IgM formulation that is stable enough to be used as a pharmaceutical.

Means to Solve the Problems

As a result of dedicated research to solve the above-mentioned problems, the present inventors discovered that, by using compounds comprising polyvalent cationic ions such as magnesium chloride and arginine hydrochloride as additives, aggregation of IgM in solutions may be suppressed and stable highly concentrated IgM solutions may be prepared.

Specifically, the present invention relates to methods for stabilizing highly concentrated IgM, solutions in which highly concentrated IgM is stabilized, and methods for preparing the solutions. More specifically, the present invention provides the following:

(1) a solution wherein a high concentration of immunoglobulin is stabilized, and wherein the immunoglobulin is IgM;

(2) the solution of (1), comprising IgM at a concentration higher than 1 mg/mL;

(3) the solution of (1), which is an aqueous solution;

(4) the solution of (1), which is a pharmaceutical formulation;

(5) the solution of (1), comprising a polyvalent cationic ion;

(6) the solution of (5), comprising the polyvalent cationic ion at a concentration of 1 mM to 1,000 mM;

(7) the solution of (5), wherein the polyvalent cationic ion is a Mg ion or an Arg ion;

(8) the solution of (5), further comprising sugars;

(9) the solution of (1), which is pH5 to pH8;

(10) the solution of (1), wherein the solution does not intrinsically comprise human-derived proteins other than IgM;

(11) the solution of (1), wherein the solution does not intrinsically comprise proteins other than IgM;

(12) a pharmaceutical formulation obtained by freezing or lyophilizing the solution of any one of (1) to (11);

(13) a method for stabilizing a solution comprising a high concentration of immunoglobulin, wherein the immunoglobulin is IgM and wherein the method comprises adding a polyvalent cationic ion to the solution;

(14) the method of (13), wherein the solution comprises IgM at a concentration higher than 1 mg/mL;

(15) the method of (13), wherein the solution is an aqueous solution;

(16) the method of (13), wherein the solution is a pharmaceutical formulation;

(17) the method of (13), which comprises adding a polyvalent cationic ion to the solution such that the solution comprises the polyvalent cationic ion at a concentration of 1 mM to 1,000 mM;

(18) the method of (13), wherein the polyvalent cationic ion is a Mg ion or an Arg ion;

(19) the method of (13), further comprising addition of sugars;

(20) the method of (13), wherein the pH of the solution is 5 to 8;

(21) the method of (13), wherein the solution does not intrinsically comprise human-derived proteins other than IgM;

(22) the method of (13), wherein the solution does not intrinsically comprise proteins other than IgM;

(23) a method for stabilizing a pharmaceutical formulation, which comprises the steps of:

(a) performing the method of any one of (13) to (22); and

(b) freezing or lyophilizing the solution stabilized in step (a);

(24) a method for producing a solution comprising a high concentration of stabilized immunoglobulin, wherein the immunoglobulin is IgM and wherein the method comprises the step of adding a polyvalent cationic ion to the solution;

(25) the method of (24), wherein the solution comprises IgM at a concentration higher than 1 mg/mL;

(26) the method of (24), wherein the solution is an aqueous solution;

(27) the method of (24), wherein the solution is a pharmaceutical formulation;

(28) the method of (24), which comprises the step of adding a polyvalent cationic ion to the solution such that the solution comprises the polyvalent cationic ion at a concentration of 1 mM to 1000 mM;

(29) the method of (24), wherein the polyvalent cationic ion is a Mg ion or an Arg ion;

(30) the method of (24), which further comprises the step of adding sugars;

(31) the method of (24), wherein the pH of the solution is 5 to 8;

(32) the method of (24), wherein the solution essentially does not comprise human-derived proteins other than IgM;

(33) the method of (24), wherein the solution essentially does not comprise proteins other than IgM;

(34) a solution which is produced by the method of any one of (24) to (33); and

(35) a method for producing a pharmaceutical formulation, wherein the method comprises the steps of:

(a) performing the method of any one of (24) to (33); and

(b) freezing or lyophilizing the solution produced in step (a).

Brief Description of the Drawings

Fig. 1 shows "Δaggregate" values obtained by subtracting the initial aggregate content from the aggregate content after one month of storage at 25°C (25°C -1 month) and the aggregate content after 2 months of storage at 25°C (25°C -2 months) for each sample.

Fig. 2 shows the aggregate content (%) in each sample in the initial state and at 4°C-3 months.

Fig. 3 shows the aggregate content (%) in each sample in the initial state, at 4°C-4 months, and 25°C-4 months.

Fig. 4 shows the aggregate content (%) according to size exclusion chromatography (SEC) in each sample in the initial state, liquid/ 40°C-8 days, liquid/ 40°C-8 days + 4°C-3 days, lyophilized/ 40°C-8 days, lyophilized/ 40°C-8 days + 4°C-3 days, and lyophilized/ 40°C-8 days + 4°C-3 days + relyophilized/50°C-8 days.

Fig. 5 shows the aggregate content (%) according to SEC in each sample in the initial state, and lyophilized/40°C-2 months.

Fig. 6 shows the chromatogram obtained by gel permeation chromatography (GPC)-MALLS analysis of MABON-01 and the molecular weight determined by calculation.

Detailed Description

In the present invention, the term "IgM" refers to an immunoglobulin that comprises constant regions of the μ chain as the constant regions of the heavy chains, and forms a pentameric or hexameric structure. The origin of the variable regions constituting the IgM of the present invention is not limited. Therefore, in addition to a variable region derived from the μ chain, the IgM of the present invention may comprise a variable region derived from IgG, or a partial structure thereof. The partial structure of a variable region can comprise the framework

and CDR. The "IgM" in the present invention refers to expression products of exogenous IgM genes introduced into cells for transformation.

Furthermore, the constant regions constituting the IgM of the present invention may be derived from any animal species. That is, the IgM of the present invention comprises an IgM constant region derived from any type of animal species carrying an IgM-type immunoglobulin. When IgM is administered *in vivo*, at least its constant regions are preferably derived from an animal species same as the species to which the IgM is administered. Therefore, when the IgM is administered to humans, at least its constant regions are preferably derived from humans. IgM composed of constant regions derived from humans, and variable regions derived from another animal species or another human, is called a chimeric antibody. A more preferable IgM for administration to humans is an IgM whose variable region framework is derived from humans, in addition to the constant regions. Human antibodies which have retained the variable region framework structure, but only the CDR has been replaced with that of an antibody from another animal species are called humanized antibodies.

In the present invention, the phrase "highly concentrated immunoglobulin (IgM)" means that the IgM content in a solution is higher than 1 mg/mL. Solutions of the present invention preferably have an IgM content of 1 mg/mL to 200 mg/mL. According to the present invention, IgM can be stabilized even at concentrations higher than 10 mg/mL (for example, 20 mg/mL or more, 25 mg/mL or more, 40 mg/mL or more, or 50 mg/mL or more).

In the present invention, when suppressing an increase of water-soluble aggregates, it is preferable to add a polyvalent cationic ion. A "polyvalent cationic ion" that may be used in the present invention is a divalent or higher valence cationic ion. For example, Mg^{++}, Ca^{++}, Zn^{++}, Fe^{++}, or a basic amino acid can be used. As basic amino acids, arginine, lysine, L-lysine L-glutamate, L-arginine L-glutamate, and such may be used. Preferably, the polyvalent cationic ion is Mg^{++} or arginine. In addition to polyvalent cationic ions, cationic ions that may be used in the present invention are monovalent cationic ions, examples being Na^{+} and K^{+}.

The concentration of cationic ions or polyvalent cationic ions added to solutions is usually 1 mM to 1,000 mM, preferably 10 mM to 500 mM, and more preferably 50 mM to 200 mM.

Solutions of the present invention may comprise sugars in addition to cationic ions or polyvalent cationic ions. Preferred sugars include trehalose, sucrose, and sorbitol.

Types of buffers that may be used in the present invention include phosphate buffers, acetate buffers, and citrate buffers.

The term "stabilization" in the present invention refers to suppressing the increase of water-soluble IgM aggregates produced during storage, and/or suppressing the increase of insoluble IgM aggregates (precipitates) produced during storage, and/or maintaining the function

of water-soluble IgM. Preferably, "stabilization" refers to suppressing the increase of water-soluble IgM aggregates produced during storage.

The term "water-soluble aggregates" in the present invention refers to water-soluble multimers such as dimers or trimers of IgM. The water-soluble aggregates can be detected, for example, by gel filtration chromatography. Stabilization of highly concentrated IgM solutions can be measured, for example, from the aggregate increase suppression rate, which can be calculated by using the following formula:

Suppression ratio of aggregate increase = (A-B)/A x 100

A: Percent increase in aggregates in highly concentrated IgM solution without polyvalent cationic ion (control)

B: Percent increase in aggregates in highly concentrated IgM solution with polyvalent cationic ion (test sample)

The aggregate increase suppression rate for the solutions of the present invention one month after addition of polyvalent cationic ions to the solution comprising a high concentration of IgM is preferably 10% or more, more preferably 30% or more, even more preferably 50% or more, and yet even more preferably 80% or more.

Solutions of the present invention are preferably those not comprising human-derived proteins other than IgM. More preferable are solutions not comprising proteins other than IgM in amounts that may make the proteins effective as stabilizers, or in amounts greater than that. When the solutions of the present invention are pharmaceutical formulations, solutions not comprising human-derived proteins other than IgM, in amounts acceptable for a pharmaceutical and/or for a bulk drug substance of a pharmaceutical are preferable, or amounts greater than that.

The dosage form of pharmaceutical formulations of the present invention is not particularly limited, and any discretionary dosage form is possible. Examples of the dosage form include a solution formulation and a lyophilized formulation. Examples of the solution formulations include formulations stored in a cold place, formulations stored at room temperature, and frozen formulations. There are no particular limitations on the administration route for the pharmaceutical formulations of the present invention; any administration route can be used. The pharmaceutical formulations may thus be administered either orally or parenterally depending on the purpose of use.

Specific dosage forms for parenteral administration include injections, and dosage forms for nasal administration, pulmonary administration, and transdermal administration. Systemic or local injections can be carried out by intravenous injections, intramuscular injections, peritoneal injections, subcutaneous injections, or such.

In addition to administering directly to patients as is, IgM stabilized by methods of the present invention can be administered as pharmaceutical agents formulated by well-known pharmaceutical methods. For example, the stabilized IgM can be used as sterile solutions prepared with water or other pharmaceutically acceptable liquid, or as injections of suspensions. Furthermore, it may be formulated by, for example, appropriately combining with pharmaceutically acceptable carriers or media, such as sterilized water, saline, emulsifiers, suspending agents, surfactants, stabilizers, vehicles, and preservatives, and mixing them at a unit dosage form required for generally accepted pharmaceutical practice. The amount of active ingredient in these formulations can be adjusted so that an appropriate dose within an indicated range can be acquired.

Sterile compositions for injections can be formulated according to usual pharmaceutical practice using vehicles such as distilled water for injections. Examples of aqueous solutions used for injections include physiological saline and isotonic solutions comprising glucose and other auxiliary agents. Specifically, the auxiliary agents may be sucrose, D-sorbitol, D-mannose, D-mannitol, sodium chloride, and such. Suitable solubilizers may also be added to pharmaceutical compositions. For example, alcohols and non-ionic surfactants are preferred solubilizers. Specific examples of alcohols comprise ethanol, polyalcohols such as propylene glycol and polyethylene glycol. Examples of non-ionic surfactants may be Polysorbate80, Polysorbate20, Poloxamer188, HCO-50, and such. Cationic surfactants such as benzalkonium chloride may also be used.

Oily fluids may be, for example, sesame oil and soybean oil, and may be used together with benzyl benzoate or benzyl alcohol as a solubilizer. Furthermore, buffers such as phosphate buffer and sodium acetate buffer, analgesic agents such as procain hydrochloride, stabilizers such as benzyl alcohol and phenol, and antioxidants may be combined. The prepared injections are usually loaded into suitable vials or ampules.

When the solutions of the present invention are made into pharmaceutical formulations, their pH is preferably 5 to 8, and particularly preferably 5 to 7.

The administration dose of the pharmaceutical formulations can be appropriately selected according to the disease to be treated, and age and symptoms of the patient. For example, a single dose can be selected within the range of 0.0001 mg to 1,000 mg per 1 kg body weight. Alternatively, for example, the dose can be selected within the range of 0.001 to 100,000 mg/body of patient. However, doses of the pharmaceutical formulations of the present invention are not limited to these. One can refer to WO 2002/096457 for the preparation of liquid formulations and such of the present invention.

All prior art literature cited herein are incorporated herein by reference.

Examples

Hereinafter, the present invention is specifically illustrated with reference to Examples, but it is not to be construed as being limited thereto.

[Example 1]

In the following Examples, recombinant anti-ganglioside GM3 human antibody produced in Reference Example 1 (hereinafter, referred to as "MABON-01") was used as the IgM. The MABON-01 solution was concentrated to prepare a highly concentrated solution of approximately 9 mg/mL. For buffer replacement, this solution was dialyzed against buffers 1 to 6 as listed below using a dialysis membrane, SLIDE-A-LYZER Dialysis Cassette 10000MWCO (PIERCE).

1. 20 mM sodium acetate, 300 mM NaCl, pH5.0/ acetate pH5.0
2. 20 mM sodium acetate, 300 mM NaCl, pH5.5/ acetate pH5.5
3. 20 mM sodium acetate, 300 mM NaCl, pH6.0/ acetate pH6.0
4. 20 mM sodium citrate, 300 mM NaCl, pH5.0/ citrate pH5.0
5. 20 mM sodium citrate, 300 mM NaCl, pH5.5/ citrate pH5.5
6. 20 mM sodium citrate, 300 mM NaCl, pH6.0/ citrate pH6.0

The resulting solutions were collected, and the concentration of MABON-01 in each sample was adjusted to 8.4 mg/mL. Each sample was placed in a storage container, Multiply-Safecup 0.1 ml Biosph. (SARSTEDT). Stability tests were carried out on these samples at 4°C. The samples were evaluated at the initial state and after storing at 4°C for 2 months (hereafter referred to as, for example "4°C–2 months"). The stability of each sample was evaluated based on changes in the residual monomer ratio determined by gel filtration chromatography. G4000SW$_{XL}$ (TOSOH) was used as the column for gel filtration chromatography. A solution comprising 50 mM sodium phosphate and 500 mM KCl (pH7.4) was used as the mobile phase. The residual monomer ratio in a sample was calculated from the values of aggregate peak area and monomer peak area obtained as a result of gel filtration chromatography. The residual monomer ratio in each sample at 4°C-2 months when the residual monomer ratio at the initial state was defined as 100% is summarized in Table 1.

[Table 1]

	Residual Monomer Ratio (%)	
pH	Acetate	Citrate
5.0	99.33	99.16
5.5	99.51	99.3

6.0	98.92	98.9

Thus stable, highly concentrated MABON-01 solutions could be prepared in citrate or acetate buffers (between pH5.0 and pH6.0) comprising 300 mM NaCl.

The following Examples describe stabilized and highly concentrated MABON-01 solutions, in which increase of water-soluble aggregates is further suppressed by adding polyvalent cations to solutions comprising citrate buffer (pH5.5).

[Example 2]

The MABON-01 solution was concentrated to prepare a highly concentrated solution of approximately 18 mg/mL. This solution was dialyzed using a dialysis membrane, SLIDE-A-LYZER Dialysis Cassette 10000MWCO (PIERCE), to replace the buffer with a solution comprising 20 mM citric acid and 300 mM NaCl (pH5.5) (the buffer type and pH were optimized under conditions without additives). This highly concentrated MABON-01 solution was dialyzed against the following buffers 1 to 9 using EasySep (TOMY) to replace the buffer.

1. 20 mM sodium citrate, 300 mM NaCl, pH5.5 / no additives
2. 20 mM sodium citrate, 900 mM NaCl, pH5.5 / NaCl
3. 20 mM sodium citrate, 300 mM NaCl, 200 mM $MgCl_2$, pH5.5 / $MgCl_2$
4. 20 mM sodium citrate, 300 mM NaCl, 200 mM Na_2SO_4, pH5.5 / Na_2SO_4
5. 20 mM sodium citrate, 300 mM NaCl, 100 mM sodium L-glutamate, pH5.5 / sodium L-glutamate
6. 20 mM sodium citrate, 300 mM NaCl, 100 mM L-arginine hydrochloride, pH5.5 / L-arginine hydrochloride
7. 20 mM sodium citrate, 300 mM NaCl, 100 mM sodium N-acetyltryptophanate, pH5.5 / sodium N-acetyltryptophanate
8. 20 mM sodium citrate, 300 mM NaCl, 10 mM urea, pH5.5 / urea
9. 20 mM sodium citrate, 300 mM NaCl, 100 mM trehalose, pH5.5 / trehalose

The resulting solutions were collected, and the concentration of MABON-01 in each sample was adjusted to 18.5 mg/mL. Each sample was placed in a storage container, Multiply-Safecup 0.1ml Biosph. (SARSTEDT). Stability tests were carried out on these samples at 25°C. The samples were evaluated at the initial state, at 25°C-1 month, and at 25°C-2 months. The stability of each sample was evaluated based on changes (increase) in aggregate content determined by gel filtration chromatography. G4000SW_{XL} (TOSOH) was used as the column for gel filtration chromatography. A solution comprising 50 mM sodium phosphate and 500 mM KCl (pH7.4) was used as the mobile phase. The aggregate content in the samples was calculated from the values of aggregate peak area and monomer peak area

obtained from gel filtration chromatography. Values obtained by subtracting the aggregate content in the initial state from the aggregate content at 25°C-1 month and at 25°C-2 months for each sample are shown in Fig. 1 as Δaggregate values.

As a result, increase of aggregates was suppressed in sample 2 [NaCl (comprising 900 mM NaCl)], when compared to sample 1 [no additives (comprising 300 mM NaCl)]. This showed that increase of aggregates can be suppressed by increasing the NaCl concentration.

Meanwhile, although samples 2 [NaCl (comprising 900 mM NaCl)], 3 [$MgCl_2$ (comprising 300 mM NaCl + 200 mM $MgCl_2$)], and 4 [Na_2SO_4 (comprising 300 mM NaCl + 200 mM Na_2SO_4)] all have the same ionic strength (each has an ionic strength of 0.9 M), $MgCl_2$ comprising a divalent cation showed a significant stabilization effect, while sample 4 [Na_2SO_4] comprising a divalent anion showed a stabilization effect similar in magnitude to that in sample 2 [NaCl].

Although sample 5 (sodium L-glutamate) and sample 6 (L-arginine hydrochloride) both comprise ionic amino acids, sample 5 comprising sodium L-glutamate did not show a stabilization effect, whereas sample 6 comprising L-arginine hydrochloride, which comprises a divalent cation, showed a stabilization effect similar to that of sample 3 ($MgCl_2$).

The results revealed that increasing the ionic strength by adding salts such as NaCl leads to aggregate suppression. Moreover, at the same ionic strength, use of divalent cations such as magnesium or arginine leads to stronger aggregate suppression effect. However, a strong aggregate suppression effect cannot be obtained by using divalent anions such as sulfate ion and glutamate. More specifically, interaction of a divalent cation such as magnesium ion or arginine with MABON-01 significantly suppressed the aggregation of MABON-01, enabling the preparation of stable and highly concentrated solutions.

[Example 3]

MABON-01 solution was concentrated to prepare a highly concentrated MABON-01 solution of approximately 19 mg/mL. The solution was dialyzed against the following buffers 1 to 9 using a dialyzer membrane, EasySep (TOMY), to replace the buffer.

1. 20 mM sodium citrate, 300 mM NaCl, pH5.5
2. 20 mM sodium citrate, 300 mM NaCl, 10 mM $MgCl_2$, pH5.5
3. 20 mM sodium citrate, 300 mM NaCl, 50 mM $MgCl_2$, pH5.5
4. 20 mM sodium citrate, 300 mM NaCl, 200 mM $MgCl_2$, pH5.5
5. 20 mM sodium citrate, 300 mM NaCl, 200 mM $MgCl_2$, 100 mM trehalose, pH5.5
6. 20 mM sodium citrate, 300 mM NaCl, 10 mM L-arginine hydrochloride, pH5.5
7. 20 mM sodium citrate, 300 mM NaCl, 50 mM L-arginine hydrochloride, pH5.5
8. 20 mM sodium citrate, 300 mM NaCl, 100 mM L-arginine hydrochloride, pH5.5

9. 20 mM sodium citrate, 300 mM NaCl, 100 mM L-arginine hydrochloride, 100 mM trehalose, pH5.5

The resulting solutions were collected, and the concentration of MABON-01 in each sample was adjusted to 18.9 mg/mL. Each sample was placed in a storage container, Multiply-Safecup 0.1ml Biosph. (SARSTEDT). Stability tests were carried out on these samples. The samples were evaluated at the initial state and at 4°C-3 months. The stability of each sample was evaluated based on changes (increases) in aggregate content determined by gel filtration chromatography. G4000SW$_{XL}$ (TOSOH) was used as the column for gel filtration chromatography. A solution comprising 50 mM sodium phosphate and 500 mM KCl (pH7.4) was used as the mobile phase. The aggregate content in the samples was calculated from the values of the aggregate peak area and the monomer peak area obtained from gel filtration chromatography. The aggregate content of each sample at the initial state and at 4°C-3 months are shown in Fig. 2.

As a result, a $MgCl_2$ and L-arginine hydrochloride concentration-dependent aggregate suppression effect was observed. More specifically, a significant stabilization effect could be obtained by increasing $MgCl_2$ and L-arginine hydrochloride concentrations. Furthermore, while trehalose alone was not effective (Examples 2 and 4), addition of 100 mM trehalose in the presence of 200 mM $MgCl_2$ or 100 mM L-arginine hydrochloride was effective for stabilization.

[Example 4]

The MABON-01 solution was concentrated to prepare a highly concentrated MABON-01 solution of approximately 27 mg/mL. The solution was dialyzed against the following buffers 1 to 3 using a dialyzer membrane, EasySep (TOMY), to replace the buffer.

1. 20 mM sodium citrate, 300 mM NaCl, pH5.5 / no additives
2. 20 mM sodium citrate, 300 mM NaCl, 200 mM $MgCl_2$, pH5.5 / $MgCl_2$
3. 20 mM sodium citrate, 300 mM NaCl, 100 mM trehalose, pH5.5 / trehalose

The resulting solutions were collected, and the concentration of MABON-01 in each sample was adjusted to 26.8 mg/mL. Each sample was placed in a storage container, Multiply-Safecup 0.1ml Biosph. (SARSTEDT). Stability tests were carried out on these samples. The samples were evaluated at the initial state, at 4°C-4 months, and at 25°C-4 months. The stability of each sample was evaluated based on changes (increases) in aggregate content determined by gel filtration chromatography. G4000SW$_{XL}$ (TOSOH) was used as the column for gel filtration chromatography. A solution comprising 50 mM sodium phosphate and 500 mM KCl (pH7.4) was used as the mobile phase. The aggregate content in the samples was calculated from the values of aggregate peak area and monomer peak area obtained from gel filtration chromatography. The aggregate content of each sample at the initial state, at 4°C-4

months, and at 25°C-4 months are shown in Fig. 3.

As a result, at both 4°C and 25°C, $MgCl_2$ was observed to have a stabilization effect. On the other hand, such an effect was hardly observed for trehalose, which is known to be a conventional protein stabilizer and which has been found to have a stabilization effect on IgM during lyophilizing as reported in Journal of Immunological Methods 1995, 181(1), 37-43 (Fig. 3).

[Example 5]

<Preparation>

A large-scale dialysis of MABON-01 was carried out in "50 mM sodium citrate, 180 mM NaCl, pH5.5, 5% sucrose" buffer, "50 mM sodium citrate, 180 mM ArgHCl, pH5.5, 5% sucrose" buffer, or "50 mM sodium citrate, 180 mM $MgCl_2$, pH5.5, 5% sucrose" buffer. After dialysis, the solutions were concentrated by filter centrifugation. Centrifugation was carried out using VIVASPIN6 5000MWCO (VIVASCIENCE, VS061) on himac CF8DL (Hitachi, No. SZGEQ054) at 3,000 rpm. After concentrating and collecting the samples, their concentrations were determined based on UV absorption ($\varepsilon = 1.40$). Next, the samples were diluted to 48.4 mg/mL using the buffers. 1% polysorbate80 solution was further added to each sample to prepare a 0.01% polysorbate formulation. 500 μL each of these samples was individually seeded into 5-mL glass vials, and 30 μL of each sample was placed in Multiply-Safecup 0.1 mL Biosph. (SARSTEDT). The 5-mL glass vials were lyophilized under the following conditions. 1 mg/mL MABON-01 was used as the initial sample, and was stored at 4°C until analysis.

Table 2

Temperature [°C]	Time [hr]
-50	24
-20	0.02
-20	18
23	2.5
23	28
30	0.25
30	10
Total	82.77

<Experimental conditions>

"50 mM sodium citrate, 180 mM NaCl, pH5.5, 5% sucrose, 0.01% polysorbate80"

"50 mM sodium citrate, 180 mM ArgHCl, pH5.5, 5% sucrose, 0.01% polysorbate80"
"50 mM sodium citrate, 180 mM $MgCl_2$, pH5.5, 5% sucrose, 0.01% polysorbate80"
MABON-01: 48.4 mg/mL
Liquid/ 40°C-8 days
Liquid/ 40°C-8 days + 4°C-3 days
Lyophilized/ 40°C-8 days
Lyophilized/ 40°C-8 days + 4°C-3 days (after reconstitution)
Lyophilized/ 40°C-8 days + 4°C-3 days (after reconstitution) + relyophilized/ 50°C-8 days

<Analysis>

After incubation, 10 μL of solutions diluted to approximately 1 mg/mL (1/50 dilution solution: 4 + 296 μL) was analyzed by SEC. "50 mM sodium citrate, 500 mM KCl, pH7.4" was used as the mobile phase (flow rate: 0.3 mL/min; detection at 280 nm or 220 nm) and SEC analysis was carried out using G4000SW_{XL} (TOSOH) (Fig. 4).

Stability of the lyophilized material was higher with ArgHCl or $MgCl_2$ than with NaCl at the same concentration, even during incubation at 50°C.

[Example 6]

<Preparation>

A large-scale dialysis of MABON-01 was carried out using "50 mM sodium citrate, 180 mM NaCl, pH5.5, 5% sucrose" and "50 mM sodium citrate, 180 mM ArgHCl, pH5.5, 5% sucrose" buffers. After dialysis, the solutions were concentrated by filter centrifugation. Centrifugation was carried out using VIVASPIN6 5000MWCO (VIVASCIENCE, VS061) on himac CF8DL (Hitachi, No. SZGEQ054) at 3,000 rpm. After concentrating and collecting the samples, their concentrations were determined based on UV absorption ($\varepsilon = 1.40$). 1% polysorbate80 solution was further added to each sample to prepare a 0.01% polysorbate formulation. The samples were diluted to obtain 50 mg/mL formulations. 300 μL each of these samples was individually seeded into three 5-mL glass vials. The 5-mL glass vials were lyophilized under the following conditions. The lyophilized formulations were stored as the initial samples at 4°C until analysis.

Table 3

Temperature [°C]	Time [hr]
-50	24
-20	0.02

-20	18
23	2.5
23	28
30	0.25
30	10
Total	82.77

<Experimental conditions>

"50 mM sodium citrate, 180 mM NaCl, pH5.5, 5% sucrose, 0.01% polysorbate80"

"50 mM sodium citrate, 180 mM ArgHCl, pH5.5, 5% sucrose, 0.01% polysorbate80"

MABON-01: 50 mg/mL

Lyophilized/ 40°C-2 months

<Analysis>

After incubation, 10 μL of solutions diluted to approximately 1 mg/mL (1/50 dilution solution: 4 + 296 μL) was analyzed by SEC. The sample obtained by using "50 mM sodium citrate, 500 mM KCl, pH7.4" was used as the mobile phase (0.3 mL/min of flow rate; detection at 280 nm or 220 nm) and SEC analysis was carried out using G4000SW_{XL} (TOSOH) (Fig. 5).

Stabilization effect was higher with ArgHCl than with NaCl at the same concentration, even after a long-term accelerated study at 40°C-2 months.

[Reference Example 1] Production of recombinant human antibodies against ganglioside GM3

1.1 Construction of anti-ganglioside GM3 human antibody heavy chain gene

A gene encoding the heavy chain of a human antibody that binds to ganglioside GM3 was amplified by RT-PCR using total RNAs extracted from human B cells transformed with Epstein-Barr virus (hereinafter, denoted as anti-ganglioside GM3 human antibody-expressing B cells).

Total RNAs were extracted from 1 x 10^7 anti-ganglioside GM3 human antibody-expressing B cells using RNeasy Plant Mini Kit (QIAGEN). Two oligonucleotides (LMH-f3 and LMH-r3) were designed based on the nucleotide sequence of anti-ganglioside GM3 human antibody gene reported by Hoon *et al.* (Cancer Research 1993; 53: 5244-5250). LMH-f3 (SEQ ID NO: 7) was synthesized in the sense direction, and LMH-r3 (SEQ ID NO: 8) was synthesized in the antisense direction. Using 1 μg of total RNAs, gene fragments were amplified separately for the 5' end and the 3' end by SMART RACE cDNA Amplification Kit (CLONTECH). Synthetic oligonucleotides LMH-r3 and LMH-f3 were used for amplifying the

5' and 3' ends of the gene, respectively. Reverse transcription reaction was carried out at 42°C for 1.5 hours.

The composition of the PCR reaction solution (50 μL) is shown below:

5 μL of 10x Advantage 2 PCR Buffer,
5 μL of 10x Universal Primer A Mix,
0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),
1μL of Advantage 2 Polymerase Mix,
(All the above were from CLONTECH)
2.5 μL of reverse transcription product, and
10 pmol of synthetic oligonucleotide LMH-f3 or LMH-r3.

The reaction was carried out under the conditions of:

94°C (initial temperature) for 30 seconds,
5 cycles of 94°C for 5 seconds and 72°C for 3 minutes,
5 cycles of 94°C for 5 seconds, 70°C for 10 seconds, and 72°C for 3 minutes,
25 cycles of 94°C for 5 seconds, 68°C for 10 seconds, and 72°C for 3 minutes, and
finally 72°C for 7 minutes.

The PCR products were purified from agarose gel using QIAquick Gel Extraction Kit (QIAGEN), and then cloned into pGEM-T Easy vector (Promega). After sequencing, an approximately 1.1 kbp fragment was obtained by digesting the vector comprising the 5' end of the gene using restriction enzymes ApaI (Takara Shuzo) and SacII (Takara Shuzo), while an approximately 1.1 kbp fragment was obtained by digesting the vector comprising the 3' end of the gene using restriction enzymes ApaI (Takara Shuzo) and NotI (Takara Shuzo). The fragments were then mixed, and cloned into pBluescript KS+ vector (TOYOBO) to obtain a full-length anti-ganglioside GM3 human antibody heavy chain gene.

To clone into vectors for expression in animal cells, full-length gene fragments were amplified using synthetic oligonucleotides LMH-fxho and LMH-rsal. LMH-fxho (SEQ ID NO: 11) is a forward primer designed to hybridize to the 5' end of the anti-ganglioside GM3 human antibody heavy chain gene, and to comprise an XhoI restriction enzyme recognition sequence and a Kozak sequence. LMH-rsal (SEQ ID NO: 12) is a reverse primer designed to hybridize to the 3' end of the anti-ganglioside GM3 human antibody heavy chain gene, and to comprise a SalI restriction enzyme recognition sequence.

The composition of the PCR reaction solution (50 μL) is shown below:

5 μL of 10x PCR Buffer,
1 mM $MgSO_4$,
0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),
1 unit of DNA polymerase KOD-Plus,
(All the above were from TOYOBO),
10 ng of pBluescript KS+ vector comprising the full-length anti-ganglioside GM3 human antibody heavy chain gene, and
10 pmol of synthetic oligonucleotides LMH-fxho and LMH-rsal.

The reaction was carried out under conditions of:
94°C (initial temperature) for 2 minutes,
30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 2 minutes, and
finally 72°C for 5 minutes.

The amplified gene fragment was cloned by digesting with the XhoI restriction enzyme (Takara Shuzo) and the SalI restriction enzyme (Takara Shuzo), then purifying using QIAquick PCR Purification Kit (QIAGEN), and linking to the XhoI restriction enzyme site of pUCAG. This pUCAG vector is obtained by: linking the 2.6 kbp fragment obtained by digesting pCXN (Niwa *et al.*, Gene 1991; 108: 193-200) using the BamHI restriction enzyme to the BamHI restriction enzyme site of pUC19 vector (TOYOBO). The obtained plasmid was named pUCAG/L612H. The nucleotide sequence and amino acid sequence of the anti-ganglioside GM3 human antibody heavy chain in this plasmid are shown in SEQ ID NOs: 1 and 2, respectively.

1.2 Construction of anti-ganglioside GM3 human antibody light chain gene

A gene encoding the light chain of anti-ganglioside GM3 human antibody was amplified by RT-PCR using total RNAs extracted from the anti-ganglioside GM3 human antibody-expressing B cells. The total RNAs were extracted from the anti-ganglioside GM3 human antibody-expressing B cells in a manner similar to that mentioned above. Two oligonucleotides (LML-f1 and LML-r1) were designed based on the nucleotide sequence of anti-ganglioside GM3 human antibody gene reported by Hoon *et al.* (Cancer Research 1993; 53: 5244-5250). LML-f1 (SEQ ID NO: 9) and LML-r1 (SEQ ID NO: 10) were synthesized in the sense and antisense directions, respectively.

Using 1 μg of total RNAs, gene fragments were amplified separately for the 5' end and the 3' end by the SMART RACE cDNA Amplification Kit (CLONTECH). Synthetic oligonucleotides LML-r1 and LML-f1 were used for amplifying the 5' and 3'ends of the gene,

respectively. Reverse transcription reaction was carried out at 42°C for 1.5 hours.

The composition of the PCR reaction solution (50 μL) is shown below:
5 μL of 10x Advantage 2 PCR Buffer,
5 μL of 10x Universal Primer A Mix,
0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),
1 μL of Advantage 2 Polymerase Mix,
(All the above were from CLONTECH)
2.5 μL of reverse transcription product, and
10 pmol of synthetic oligonucleotide LML-f1 or LML-r1

The reaction was carried out under conditions of:
94°C (initial temperature) for 30 seconds,
5 cycles of 94°C for 5 seconds and 72°C for 3 minutes,
5 cycles of 94°C for 5 seconds, 70°C for 10 seconds, and 72°C for 3 minutes,
25 cycles of 94°C for 5 seconds, 68°C for 10 seconds, and 72°C for 3 minutes, and
finally 72°C for 7 minutes.

PCR product was purified from the agarose gel using QIAquick Gel Extraction Kit (QIAGEN), and then cloned into pGEM-T Easy vector (Promega). After sequencing, an approximately 0.7 kbp fragment was obtained by digesting the vector comprising the 5' end of the gene using the EcoRI restriction enzyme (Takara Shuzo), while an approximately 0.9 kbp fragment was obtained by digesting the vector comprising the 3' end of the gene using the EcoRI restriction enzyme (Takara Shuzo). The two fragments were mixed, and used to amplify the full-length gene fragment using synthetic oligonucleotides LML-feco and LML-rnot. LML-feco (SEQ ID NO: 13) is a forward primer, and was designed to hybridize to the 5' end of the anti-ganglioside GM3 human antibody light chain gene, and to comprise an EcoRI restriction enzyme recognition sequence and a Kozak sequence. LML-rnot (SEQ ID NO: 14) is a reverse primer, and was designed to hybridize to the 3' end of the anti-ganglioside GM3 human antibody light chain gene, and to comprise a NotI restriction enzyme recognition sequence.

The composition of the PCR reaction solution (50 μL) is shown below:
5 μL of 10x PCR Buffer,
1 mM $MgSO_4$,
0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),
1 unit of DNA polymerase KOD-Plus,

(All the above were from TOYOBO)

5'-end gene fragment,

3'-end gene fragment, and

10 pmol of synthetic oligonucleotides LML-feco and LML-rnot.

The reaction was carried out under conditions of:

94°C (initial temperature) for 2 minutes,

30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 2 minutes, and

finally 72°C for 5 minutes.

The amplified gene fragment was cloned by digesting with the EcoRI restriction enzyme (Takara Shuzo) and the NotI restriction enzyme (Takara Shuzo), then purifying using QIAquick PCR Purification Kit (QIAGEN) and linking to the EcoRI and NotI restriction enzyme cleavage sites of pCXND3.

The pCXND3 vector was constructed as follows: DHFR-ΔE-rvH-PM1-f (see WO 92/19759) was digested at the EcoRI/SmaI restriction enzyme site to separate their antibody heavy chain gene and vector region. Only the vector portion was then collected, into which the EcoRI-NotI-BamHI adaptor (Takara Shuzo) was cloned. This vector was named pCHOI.

A vector in which the DHFR gene expression site of pCHOI is cloned into the HindIII restriction enzyme site of pCXN (Niwa *et al.*, Gene 1991; 108:193-200) was named pCXND3. Furthermore, the light-chain gene fragment was cloned into pCXND3 and the obtained plasmid was named pCXND3/L612L. The nucleotide sequence and amino acid sequence of anti-ganglioside GM3 human antibody light chain in this plasmid are shown in SEQ ID NOs: 3 and 4, respectively.

1.3 Construction of the anti-ganglioside GM3 human antibody expression vector

To produce the anti-ganglioside GM3 human antibody expression vector, pUCAG/L612H was digested with the HindIII restriction enzyme (Takara Shuzo), and the resulting approximately 4.0 kbp fragment was linked to the HindIII restriction enzyme cleavage site of pCXND3/l612L. The obtained plasmid was named pCXND3/L612IgM. This plasmid expresses the neomycin-resistance gene, DHFR gene, and anti-ganglioside GM3 human antibody gene in animal cells.

1.4 Construction of anti-ganglioside GM3 human antibody J-chain gene and expression vector

A gene encoding the J chain of anti-ganglioside GM3 human antibody was amplified by RT-PCR using total RNAs extracted from anti-ganglioside GM3 human antibody-expressing B

cells. Total RNAs were extracted from anti-ganglioside GM3 human antibody-expressing B cells in a manner similar to that mentioned above. Two oligonucleotides (J-f1 and J-r1) were designed and synthesized based on the nucleotide sequence of the human antibody J chain gene registered in GenBank (GenBank accession number: M12759). J-f1 (SEQ ID NO: 15) hybridizes to human antibody J chain gene Exon 3 in the sense direction, and J-r1 (SEQ ID NO: 16) hybridizes to the human antibody J chain gene Exon 4 in the antisense direction.

Using 1 μg of total RNAs, gene fragments were amplified separately for the 5' end and the 3' end by the SMART RACE cDNA Amplification Kit (CLONTECH). Synthetic oligonucleotides J-r1 and J-f1 were used for amplifying the 5' and 3' ends of the gene, respectively. Reverse transcription reaction was carried out at 42°C for 1.5 hours.

The composition of the PCR reaction solution (50 μL) is shown below:

5 μL of 10x Advantage 2 PCR Buffer,
5 μL of 10x Universal Primer A Mix,
0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),
1 μL of Advantage 2 Polymerase Mix,
(All the above were all from CLONTECH)
2.5 μL of reverse transcription product, and
10 pmol of synthetic oligonucleotide J-f1 or J-r1

The reaction was carried out under conditions of:

94°C (initial temperature) for 30 seconds,
5 cycles of 94°C for 5 seconds and 72°C for 3 minutes,
5 cycles of 94°C for 5 seconds, 70°C for 10 seconds, and 72°C for 3 minutes,
25 cycles of 94°C for 5 seconds, 68°C for 10 seconds, and 72°C for 3 minutes, and finally 72°C for 7 minutes.

PCR product was purified from the agarose gel using QIAquick Gel Extraction Kit (QIAGEN), and then cloned into pGEM-T Easy vector (Promega).

After sequencing, an approximately 0.5 kbp fragment was obtained by digesting the vector comprising the 5' end of the gene using the EcoRI restriction enzyme (Takara Shuzo), and an approximately 1.0 kbp fragment was obtained by digesting the vector comprising the 3' end of the gene using the EcoRI restriction enzyme (Takara Shuzo). The two fragments were mixed, and used to amplify the full-length gene fragment using synthetic oligonucleotides J-feco and J-rxba.

J-feco (SEQ ID NO: 17) is a forward primer designed to hybridize to the 5' end of the

anti-ganglioside GM3 human antibody J chain gene, and to comprise an EcoRI restriction enzyme recognition sequence and a Kozak sequence. J-rxba (SEQ ID NO: 18) is a reverse primer designed to hybridize to the 3' end of the anti-ganglioside GM3 human antibody J chain gene, and to comprise an XbaI restriction enzyme recognition sequence.

The composition of the PCR reaction solution (50 μL) is shown below:

5 μL of 10x PCR Buffer,
1 mM $MgSO_4$,
0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),
1 unit of DNA polymerase KOD-Plus
(the above-mentioned ingredients were all from TOYOBO),
5'-end gene fragment,
3'-end gene fragment, and
10 pmol of synthetic oligonucleotides LML-feco and LML-rxba

The reaction was carried out under conditions of:

94°C (initial temperature) for 2 minutes,
30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 2 minutes, and
finally 72°C for 5 minutes.

The amplified gene fragment was cloned by digesting with the EcoRI restriction enzyme (Takara Shuzo) and the XbaI restriction enzyme (Takara Shuzo), then purifying using QIAquick PCR Purification Kit (QIAGEN), and linking to the EcoRI and XbaI restriction enzyme cleavage sites of pCOSII-Zeo.

This pCOSII-Zeo vector is obtained by removing the DHFR gene expression site of pCHOI, and cloning the Zeocin-resistant gene expression site thereto. The obtained plasmid was named pCOSII-Zeo/ J chain. The nucleotide sequence and amino acid sequence of anti-ganglioside GM3 human antibody J chain in this plasmid are shown in SEQ ID NOs: 5 and 6, respectively.

1.5 Expression of anti-ganglioside GM3 human antibody using animal cells

Stable expression cell lines derived from CHO cells (DG44 line) were produced as described below.

Genes were introduced by electroporation using Gene Pulser II (BioRad).

Introduction of genes to obtain cell lines that do not express the J chain is described below. 0.75 mL of CHO cells suspended in PBS (1×10^7 cells/mL) was mixed with

anti-ganglioside GM3 human antibody expression vector pCXND3/L612IgM (25 μg), cooled on ice for 10 minutes, transferred to a cuvette, and then pulsed at 1.5 kV and 25 μFD.

After a recovery period of 10 minutes at room temperature, the electroporated cells were suspended in 40 mL of CHO-S-SFMII medium (Invitrogen) comprising 1x HT Supplement (Invitrogen). A 50-fold diluted solution was further prepared using the same medium, and then aliquoted at 100 μL/well into a 96-well culture plate. After incubation for 24 hours in a CO_2 incubator (5% CO_2), Geneticin (Invitrogen) was added to the wells at 0.5 mg/mL and cultured for 2 weeks.

The IgM levels in the culture supernatants of wells in which colonies of Geneticin-resistant transformants were found were measured by the concentration assay described in Reference Example 1.6. Cell lines highly expressing the anti-ganglioside GM3 human antibodies were successively expanded to obtain anti-ganglioside GM3 human antibody-expressing stable cell lines CA02, CA15, CA19, CA20, and CA24.

Introduction of genes to obtain cell lines expressing the J chain is described below. 0.75 mL of CHO cells suspended in PBS (1 x 10^7 cells/mL) was mixed with anti-ganglioside GM3 human antibody expression vector pCXND3/L612IgM (25 μg) and J chain expression vector pCOSII-Zeo/J chain (20 μg), cooled on ice for 10 minutes, transferred to a cuvette, and then pulsed at 1.5 kV and 25 μFD.

After recovered for 10 minutes at room temperature, the electroporated cells were suspended in 40 mL of CHO-S-SFMII medium (Invitrogen) comprising 1x HT Supplement (Invitrogen).

A 50-fold diluted solution was further prepared using the same medium and aliquoted at 100 μL/well into a 96-well culture plate. After incubation for 24 hours in a CO_2 incubator (5% CO_2), 0.5 mg/mL Geneticin (Invitrogen) and 0.6 mg/mL Zeocin (Invitrogen) were added to wells, and cultured for 2 weeks. The IgM levels in the culture supernatants of wells in which colonies of Geneticin- and Zeocin-resistant transformants were found were measured by the concentration assay described in Reference Example 1.6. Cell lines highly expressing the anti-ganglioside GM3 human antibodies were successively expanded to obtain anti-ganglioside GM3 human antibody-expressing stable cell lines (CJ15, CJ25, CJ38, CJ45, and CJ67).

1.6 Measurement of IgM concentration in culture supernatants

IgM concentration in the culture supernatants was measured as described below. Anti-Human IgM (BIOSOURCE) was diluted using a coating buffer (0.1 M $NaHCO_3$ and 0.02% NaN_3) to prepare a 1 μg/mL solution. The diluted solution was added to a 96-well ELISA plate at 100 μL/well, and then reacted at 4°C for 24 hours or longer to coat the plate.

After washing the wells with Rinse Buffer, blocking was carried out by adding 200

μL/well of Diluent Buffer and reacting at room temperature for 1 hour or longer. Compositions of the Rinse Buffer and Diluent Buffer are shown below.

Rinse Buffer: PBS(-)
0.05% Tween20

Diluent Buffer: 50 mM Tris,
1 mM $MgCl_2$,
0.15 M NaCl,
0.05% Tween20,
0.02% NaN_3,
1% BSA

Next, culture supernatant suitably diluted with Diluent Buffer was added to the wells at 100 μL/well, and allowed to react at room temperature for 1 hour. After washing with Rinse Buffer, alkaline phosphatase-conjugated goat anti-human IgM (BIOSOURCE) diluted 4,000 times with Diluent Buffer was added at 100 μL/well, and reacted at room temperature for 1 hour. Finally, wells were washed with Rinse Buffer, and alkaline phosphatase substrate (SIGMA) was added thereto. The absorbance was determined at the 405 nm measurement wavelength and 655 nm reference wavelength using Benchmark Plus absorption spectrometer (BioRad). The concentration of IgM was calculated by comparing with a purified anti-ganglioside GM3 human antibody (Hoon *et al.*, Cancer Research 1993; 53: 5244-5250).

Each type of stable cell line expressing anti-ganglioside GM3 human antibodies was cultured in a 75 cm^2-culture flask at an initial cell density of 2 x 10^5 cells/mL. The IgM concentration in the culture supernatants was measured by the method described above. The results are shown in Table 4. The amount of IgM produced was approximately 20 mg/L on the third day and approximately 50 mg/L on the seventh day. The productivity indicating the production ability of a single cell was 5 to 19 pg/cell/day. Since IgM is a type of immunoglobulin that forms multimers, expression level of IgM in recombinants is low, and therefore, its large-scale preparation was considered difficult. However, the present results showed that highly productive recombinant IgM-expressing cells can be produced from CHO cells.

Table 4

J-chain expression	Cell lines	Production amount after culturing for 3 days (mg/L)	Production amount after culturing for 7 days (mg/L)	Productivity (pg/cell/day)
Absent	CA02	24.1	36.9	14.1
	CA15	11.8	39.7	4.9

	CA19	27.1	62.3	13.1
	CA20	20.2	35.4	10.5
	CA24	25.0	41.5	10.7
Present	CJ15	29.4	N.T.	19.4
	CJ25	24.4	N.T.	18.1
	CJ38	14.9	N.T.	12.4
	CJ45	26.4	N.T.	18.7
	CJ67	18.0	N.T.	12.8

N.T.: Not Tested

[Reference Example 2] Measurement of aggregates (1)

Gel filtration chromatographic analysis of MABON-01 was carried out using the following buffers as mobile phase. In all analyses, a TSKgel G4000SW$_{XL}$ column was used, the flow rate was 0.3 mL/min, absorbance at 280 nm was detected, and the injection amount of samples was 10 μg.

1. 50 mM sodium phosphate, 500 mM KCl, pH6.2
2. 50 mM sodium phosphate, 500 mM KCl, pH6.5
3. 50 mM sodium phosphate, 500 mM KCl, pH6.8
4. 50 mM sodium phosphate, 500 mM KCl, pH7.1
5. 50 mM sodium phosphate, 500 mM KCl, pH7.4
6. 50 mM sodium phosphate, 300 mM KCl, pH6.5
7. 50 mM sodium phosphate, 300 mM KCl, pH7.4
8. 50 mM sodium phosphate, 500 mM NaCl, pH6.5
9. 50 mM sodium phosphate, 500 mM NaCl, pH7.4
10. 50 mM sodium phosphate, 300 mM NaCl, pH6.5
11. 50 mM sodium phosphate, 300 mM NaCl, pH7.4

The aggregate peak area and the monomer peak area values (peak assignments were carried out separately) based on the obtained chromatograms are shown in Table 5.

Table 5

	KCl				NaCl			
	500 mM		300 mM		500 mM		300 mM	
pH6.2	3073386		–		–		Total peak area value	
	146342	2927044	–	–	–	–	Aggregate	Monomer

pH6.5	3096904		2959509		3044989		2818198	
	155304	2941600	124880	2834629	127467	2917522	82928	2735270
pH6.8	3074760		–		–		–	
	153682	2921078	–	–	–	–	–	–
pH7.1	3033846		–		–		–	
	154085	2879761	–	–	–	–	–	–
pH7.4	3074597		3098757		3130093		2948932	
	163747	2910850	157320	2941437	144630	2985463	112427	2836505

In each cell, the total peak area value is shown in the top row, the area value for the aggregate is shown in the bottom row on the left, and the area value for the monomer is shown in the bottom row on the right.

Table 6 shows the result of calculating the aggregate peak area rate and the monomer peak area rate relative to the total peak area value.

Table 6

	KCl				NaCl			
	500 mM		300 mM		500 mM		300 mM	
pH6.2	4.8	95.2	–	–	–	–	Aggregate	Monomer
pH6.5	5.0	95.0	4.2	95.8	4.2	95.8	2.9	97.1
pH6.8	5.0	95.0	–	–	–	–	–	–
pH7.1	5.1	94.9	–	–	–	–	–	–
pH7.4	5.3	94.7	5.1	94.9	4.6	95.4	3.8	96.2

In each cell, the aggregate peak area rates are shown on the left, and the monomer peak area rates are shown on the right.

These results revealed that, for both the total peak area values and the aggregate peak area rates, the effects of salt type and salt concentration are suppressed when a pH7.4 mobile phase buffer is used, compared to when mobile phase buffers of pH6.2 to 7.1 are used. In addition, it was revealed that the effect of pH is suppressed when the salt concentration in the mobile phase buffer comprising either KCl or NaCl is 500 mM, compared to when the salt concentration is 300 mM. Furthermore, it was revealed that the effects of salt concentration and mobile phase pH are suppressed when a mobile phase buffer comprises KCl, compared to when a mobile phase buffer comprises NaCl. Therefore, conditions for the mobile phase for the gel filtration chromatographic analysis of MABON-01 were set at 50 mM sodium phosphate,

500 mM KCl, and pH7.4.

[Reference Example 3] Measurement of aggregates (2)

GPC-MALLS analysis of MABON-01 was carried out to determine the molecular weight of each peak. The mobile phase buffer was 50 mM sodium phosphate, 500 mM KCl, pH7.4. TSKgel G4000SW$_{XL}$ column was used. The flow rate was 0.3 mL/min. Absorbance at 280 nm was detected. Injection amount of samples was 113 μg. According to the obtained results, the molecular weights were calculated by the Debye method.

Resulting chromatogram and the calculated molecular weights are shown as a superimposed diagram in Fig. 6. The average molecular weights of peak 1 and peak 2 in the figure, and the theoretical molecular weight of MABON-01 calculated from its amino acid sequence are shown in Table 7.

Table 7

Average molecular weights	
	M.W. [kDa]
Peak 1	1,109
Peak 2	2,193

Theoretical molecular weights	
	M.W. [kDa]
Monomer	1,034
Dimer	2,068

The average molecular weights were obtained by averaging the molecular weights corresponding to 21.5 to 22.0 minutes for peak 2, and 24.5 to 25.0 minutes for peak 1. The average molecular weight values for peak 1 and peak 2 are close to the theoretical molecular weight values for the monomer and dimer, respectively. In addition, the average molecular weight value for peak 2 is approximately two times that of peak 1. Accordingly, peak 1 was found to comprise the MABON-01 monomer, and peak 2 was found to comprise the dimer.

Industrial Applicability

The present invention enabled stabilization of highly concentrated IgM in solutions. Since the present invention enables stable long-term storage of pharmaceutical formulations comprising IgM as an active ingredient, it can significantly contribute to particularly the preparation of antibody formulations.